From Vessel Sprouting to Normalization. Role of the Prolyl Hydroxylase Domain Protein/Hypoxia-Inducible Factor Oxygen-Sensing Machinery

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Arterioscler Thromb Vasc Biol published online Oct 21, 2010;
DOI: 10.1161/ATVBAHA.110.214106
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association.
7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 1079-5642. Online
ISSN: 1524-4636

From Vessel Sprouting to Normalization

Role of the Prolyl Hydroxylase Domain Protein/Hypoxia-Inducible Factor Oxygen-Sensing Machinery

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Abstract—The accepted model of vessel branching distinguishes several endothelial cell fates. At the forefront of a vessel sprout, "tip cells" guide the sprouting vessel toward an angiogenic stimulus. Behind the tip, "stalk cells" proliferate to elongate the vessel branch and create a lumen. In mature vessels, endothelial cells acquire a streamlined shape to optimally conduct blood flow. For this purpose, endothelial cells switch to the "phalanx" cell fate, which is characterized by quiescent and nonproliferating cells aligned in a tight cobblestonelike layer. Vessel maturation also requires the recruitment of mural cells (ie, smooth muscle cells and pericytes). These cell fates are often altered in pathological conditions, most prominently during the formation of tumor vasculature. Given the essential role of hypoxia as the driving force for initiating angiogenesis, it is not surprising that the hypoxia-sensing machinery controls key steps in physiological and pathological angiogenesis. **(*Arterioscler Thromb Vasc Biol*. 2011;31:00-00.)**

Key Words: angiogenesis ■ hypoxia ■ HIF ■ PHD

Angiogenesis is a dynamic process that is tightly regulated by oxygen-sensing mechanisms. The prolyl hydroxylase domain proteins (PHDs) are oxygen-sensing enzymes that belong to the evolutionarily conserved superfamily of iron-containing 2-oxoglutarate–dependent dioxygenases. This family consists of 3 PHDs (PHD1–3) and the factor inhibiting hypoxia-inducible factor (HIF) (FIH).[1] Under well-oxygenated conditions, PHDs hydroxylate conserved prolyl residues of the HIF-α subunits, thereby generating a binding site for the von Hippel–Landau tumor suppressor protein, which targets HIF-α for proteosomal degradation. When oxygen levels decrease, the hydroxylation activity of the PHDs is reduced, leading to HIF-α accumulation. On binding to HIF-β, the HIF-α/β complex translocates and activates transcription of numerous genes, including those regulating survival, metabolism, and angiogenesis.[2] In normoxia, the transcriptional activity of HIF is reduced by hydroxylation of a conserved asparagine residue by FIH.[3] Emerging evidence suggests that HIF-1α and HIF-2α have distinct nonoverlapping roles in angiogenesis.[4] PHD2 is a key family member and, consistent with this report, its deficiency disrupts development.[5,6] Oxygen-independent pathways for the regulation of HIFs and PHDs also exist.[1,7] Moreover, the oxygen sensors can hydroxylate other proteins, some of which are associated with angiogenesis.[1] Important knockout phenotypes of these PHD/HIF molecules in mice are listed in the Table.

Oxygen Sensing in the Endothelial Sprout

Vessel branching is regulated by growth factors[8,9] and guidance cues.[10,11] Gradients of some of these signals are sensed by the tip cell, which translates them into directional migration. Tip cells are polarized, have numerous filopodia that probe the environment while the cell migrates toward an angiogenic stimulus, and do not form a lumen.[8] They have a specific molecular signature, characterized by the expression of vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2), VEGFR3, platelet-derived growth factor-β, Unc5B, neuropilin, deltalike ligand-4 (Dll4), CXCR4, and Ephrin-B2,[12–15] among other markers.[16] Dll4 binds to the Notch1 receptor of neighboring cells, thereby inducing a stalk cell phenotype.[17] Stalk cells trail behind the tip, proliferate, elongate the stalk, form a lumen, and connect to the circulation. They do not extend filopodia but rather form tight junctions and prevent endothelial cell (EC) retraction.[16]

Although hypoxia is a strong stimulus for angiogenesis, it remains unclear how hypoxia-induced signaling regulates the various steps of vessel branching. Hypoxia and HIF-1α enhance the expression of VEGFR2,[8] which induces Dll4 expression in the tip cell. Components of the oxygen-sensing machinery also modulate the Notch signaling pathway. For instance, HIF-1α interacts with the Notch intracellular domain and increases its activity.[18] Moreover, HIFs stimulate transcription of the Notch targets Hey1, Hey2, and Dll4,[19,20] whereas HIF-1α heterozygosity decreases the levels of acti-

Received on: August 1, 2010; final version accepted on: October 7, 2010.
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Arterioscler Thromb Vasc Biol **is available at http://atvb.ahajournals.org** **DOI: 10.1161/ATVBAHA.110.214106**

Table. Summary of Phenotypes in Knockout Mice of PHD/HIF Oxygen-Sensing Molecules

Gene	Genotype	Phenotype
PHD1	−/−	Hypoxia tolerance of skeletal muscle and liver due to reprogramming to anaerobic metabolism[72,73] and decreased cyclin D1 levels and mammary gland proliferation[74]
PHD2	+/−	Normal vascular development; tumor vessel normalization, leading to reduced metastasis and tumor invasiveness due to improved tumor oxygenation[30]
	−/− (Prenatal)	Embryonic lethality due to placental and heart defects[5]
	−/− (Postnatal)	Increased angiogenesis[75] and polycythemia and congestive heart failure[76]
	Silencing in tumor cells	Increased tumor growth and angiogenesis[42]
	Endothelial haplodeficiency	Tumor vessel normalization (phenocopy of the PHD2$^{+/-}$ phenotype)[30]
PHD3	−/−	Hypofunctional sympathoadrenal system and reduced blood pressure[77]
PHD1; *PHD3*	−/−;−/−	Viable and fertile with smaller litters and moderate erythrocytosis[43]
PHD3; *PHD2*	−/−;−/− (Postnatal)	Premature lethality due to hepatic steatosis and dilated cardiomyopathy[78]
FIH	−/−	Viable, but reduced, body mass; enhanced metabolic rate; and insulin sensitivity[79]
HIF-1α	+/−	Protection against vascular remodeling during pulmonary hypertension and impaired physiological response to chronic hypoxia[80]
	Endothelial inactivation	Impaired tumor angiogenesis[25]
	HIF-1α$^{KI/+}$:HIF-2α knocked in *HIF-1α* allele	Viable and decreased incidence of thymic lymphomas in a p53 mutant mouse model[21]
	HIF1α$^{KI/KI}$:HIF-2α knocked into both *HIF-1α* alleles	Embryonic lethality and embryonic stem cells generate more proliferative and vascularized teratomas[21]
	−/−	Embryonic lethality due to (cardio)vascular defects and impaired tumor angiogenesis[26,27,81–83]
HIF-2α	+/−	Viable and fertile, protection against hypoxia-induced pulmonary vasculature remodeling,[84] and reduced retinal neovascularization after oxygen-induced retinopathy[85]
	Endothelial inactivation	Defects in vessel integrity and tumor neovascularization[41]
	−/− (Postnatal)	Anemia[86]
	−/− (Prenatal)	Background-dependent phenotype: embryonic lethality due to vascular defects and vascular disorganization in yolk sac and embryo (CD1 and ICR/129Sv outbred)[35,87]; minimal viability due to multiple organ pathological features, metabolic abnormalities, and hematopoietic defects; defective catecholamine metabolism and bradycardia (C57/Black6 and 129 interbred)[37–39]; defects in lung maturation; and acute respiratory distress (129/Sv x Swiss)[36]
HIF-1α;; *HIF-2α*	−/−;−/−	Embryonic lethality due to defective placental vascularization[88]
HIFdn	Endothelial dominant negative	Embryonic lethality due to cardiovascular defects and an immature and disorganized vascular network[89]
HIF-3α (IPAS splice variant)	Oligo antisense in the cornea	Induced angiogenesis[90]
HIF-3α (NEPAS splice variant)	−/−	Enlargement of the right ventricle and impairment of lung remodeling[91]
HIF-1β	Endothelial-specific inactivation	Partial embryonic lethality due to hepatic vascular defects and impaired energy homeostasis; and liver lesions, impaired lipid serum homeostasis, and hyperactive metabolism in adulthood[92]
	−/−	Embryonic lethality, insufficient angiogenesis and bronchial arch development, defective placental vascularization and vascular development, and altered placental cell fate determination[93–96]

ICR indicates ●●●; IPAS, ●●●; NEPAS, ●●●.

vated Notch1 and its target genes *Dtx1* and *Nrarp*.[21] FIH-1 hydroxylates ankyrin repeat domains in Notch receptors, thereby decreasing their activity and the ability of FIH to hydroxylate HIFs.[22,23]

Several genetic studies suggest a role for HIF-1α in vessel sprouting. Loss of HIF-1α in ECs attenuates the expression of hypoxia-inducible angiogenic genes and reduces EC proliferation under hypoxic conditions.[24,25] EC-specific HIF-1α–deficient mice do not exhibit an overt phenotype in baseline conditions but show reduced tumor growth and vascularization because of attenuated endothelial expression of VEGF and VEGFR2. HIF-2α levels are not upregulated by the absence of HIF-1α, suggesting that HIF-2α differentially regulates EC physiological features in vivo.[25] This is in line with previous reports[26,27] that embryos lacking HIF-1α display vascular defects that are not rescued by HIF-2α. Conversely, HIF-1α stimulates neovascularization and improves functional recovery of ischemic tissues in adults.[28] Thus, although HIF-1α controls vessel sprouting, HIF-2α seems to mediate the maintenance of the newly formed sprout.

Oxygen Sensing and Endothelial Quiescence

Once a functional branch is established and is ready to fuse, the explorative behavior of the tip cell and the proliferative

nature of the stalk cell need to be suppressed. Recent studies report the involvement of HIF/PHD oxygen sensors in vessel stabilization. Angiopoietin-1 induces normalization of immature vessels, a process associated with reduced PHD2 expression.[29] Via upregulation of HIF-2α, haplodeficiency of PHD2 also induces tumor vessel normalization.[30] PHD2 haplodeficiency in tumor ECs does not affect vessel density, area, or lumen size; however, it "normalizes" the endothelial lining, barrier, and stability. This does not affect primary tumor growth. It does improve tumor oxygenation and establish a tight impenetrable endothelial barrier, which prevents a metastatic switch and impedes intravasation of tumor cells and metastasis, overall resulting in prolonged survival of tumor-bearing mice. Gene profiling revealed a lower induction of HIF-1α–dependent metastatic genes and an upregulation of the junctional protein VE-cadherin and the VEGF-trap soluble VEGFR1.[30] VE-cadherin has induced a normalized endothelial phenotype indirectly by inhibiting VEGF-driven proliferation and survival.[31,32] It also inhibits proliferation of ECs in cooperation with soluble VEGFR1 to induce EC quiescence.[33] Consistently, its expression is specifically upregulated by HIF-2α but not by HIF-1α.[34]

Genetic HIF-2α–deficient studies show variable phenotypes depending on the genetic background. In a CD1 strain, loss of HIF-2α causes improper remodeling of nascent vessels into larger conduits,[35] whereas in other backgrounds, loss of HIF-2α causes abnormal organ development or homeostasis without apparent vascular deficits.[36–39] Silencing HIF-2α by approximately 50% in host mice leads to the formation of an aberrant vascular network in tumors through reduced expression of Ephrin-A1 in ECs. This is not because of reduced sprouting vessels or hypoxic EC proliferation; rather, it is because of impaired remodeling of the microvasculature.[40] Consistently, HIF-2α deficiency in ECs results in increased vessel permeability and defective tumor neovascularization by decreasing the expression of genes involved in adhesion and extracellular matrix formation and angiogenesis.[41] Notably, loss of HIF-2α in ECs reduces metastasis (possibly because primary tumor growth was also reduced),[41] whereas HIF-2α upregulation in PHD2-haplodeficient mice also reduces metastasis (via the previously mentioned mechanism of tumor vessel normalization).[30] In contrast, PHD2 inactivation in tumor cells accelerates tumor growth via HIF-independent NF-κB pathway signaling.[42] Also, complete inactivation of PHD2 in mice after birth stimulates the outgrowth of supernumerary vessels in healthy organs,[43] indicating that the effects of PHD2 in the vasculature are dose dependent.

Bone Marrow–Derived Progenitors, Oxygen Sensing, and Angiogenesis

Recruitment of a heterogeneous population of bone marrow–derived cells (BMDCs), containing progenitors of the endothelial, pericyte, and hematopoietic lineage, may also contribute to vessel formation and maturation.[44] Some of the signals that regulate the mobilization and homing of BMDCs to hypoxic areas have been characterized. VEGFR1 and its ligands (ie, VEGF and PlGF) induce BMDC mobilization and recruitment to ischemic regions[45–47] in response to HIF-1α stabilization.[48,49] By binding CXCR4-positive BMDCs, stromal cell–derived factor-1 (SDF-1/CXCL12) promotes recruitment and retention of endothelial progenitor cells in ischemic tissues. HIF-1α upregulates CXCR4 receptor expression and enhances SDF-1 expression in ECs, thereby promoting adhesion, migration, and retention of CXCR4$^+$ BMDCs.[13,50] The inhibition of SDF-1 in ischemic tissue or of CXCR4 in circulating cells prevents endothelial progenitor cell recruitment to sites of injury.[51] HIF-1α–dependent induction of SDF-1 was documented in various angiogenesis models.[52,53] Interestingly, hypoxic BM regions also exhibit increased SDF-1 expression and endothelial progenitor cell retention, suggesting that tissue hypoxia may constitute a more general mechanism governing stem cell retention.[51]

HIF-1α–dependent induction of SDF-1 is also capable of recruiting bone marrow–derived CD45$^+$ cells of the monocytic lineage.[54] Monocyte-derived cells secrete matrix metalloproteases, which contribute to angiogenesis by degrading basement membranes and extracellular matrix components, thereby enhancing the release of proangiogenic factors from the extracellular matrix.[55–58] In addition, macrophages release the peptide PR39, which inhibits HIF-1α degradation, amplifying the hypoxic response.[59] Hypoxic induction of matrix metalloproteases contributed to cancer cell metastasis by degrading extracellular matrix components.[60–66] Development of hypoxia during antiangiogenic treatment may trigger recruitment of BDMCs, which can contribute to revascularization after treatment cessation. Interestingly, recent evidence links reduced tumor vascularization and growth during chemotherapy with a reduction in BMDC mobilization. Anthracyclines inhibit HIF-1α activity by disrupting its binding to DNA, thereby decreasing levels of HIF-1α target genes (eg, VEGF, SDF-1α, and stem cell factor).[67]

Mural Cells, Oxygen Sensing, and Vessel Maturation

Mural cells (ie, pericytes in capillaries and vascular smooth muscle cells in larger blood vessels) provide vessel stabilization and regulate vessel perfusion.[68] In PHD2-haplodeficient mice, the reduction of PHD2 levels in ECs promotes pericyte coverage of tumor ECs via HIF-2α–dependent mechanisms, thereby rendering the vessels more mature, tight, and stable.[30] In another experimental setting, inhibition of HIF-1α reduces mural cell association with tumor ECs.[69] Furthermore, noise-induced mechanical and metabolic stress causes local hypoxia in the cochlear microvasculature, which promotes the stabilization of HIF-1α. The subsequent upregulation of VEGF in both pericytes and ECs results in exaggerated pericyte coverage and abnormal vessel morphological features, yielding leaky and dysfunctional vessels.[70] Another link between oxygen sensing and vessel maturation may be platelet-derived growth factor receptor-β signaling, which controls mural cell recruitment and can be suppressed by VEGF-induced activation of VEGFR2.[71]

Conclusions

An important trigger activating the formation of a new blood vessel is the lack of oxygen in surrounding tissues. Therefore, it is not surprising that ECs and other cells composing the mature vessel are able to sense oxygen. Although previous

research has mainly focused on the role of VEGF, evidence is emerging that oxygen levels inside the vessel wall itself determine the fate of the developing sprout. Therefore, exploring the role of HIF-α, a master modulator of the hypoxic response, and its regulators (ie, PHD oxygen sensors) is crucial to improving our understanding of angiogenesis. Inside the endothelium, HIF-1α is presumed to control sprouting, whereas HIF-2α, in a dose-dependent way, ensures vessel quiescence and stability. In mural cells, a similar dose dependency also seems to determine the HIF-1α–mediated response to hypoxia; however, it is still unclear whether HIF-2α is also involved in conferring adequate pericyte or smooth muscle cell function. In addition, HIF-1α has triggered the recruitment of progenitor cells that could also contribute to vessel stabilization.

The knowledge that the oxygen machinery influences all players conferring a mature functional vessel has broad clinical implications. Increasing tumor hypoxia through antiangiogenic therapy induces the PHD/HIF oxygen-sensing machinery that is likely to reestablish the vasculature as soon as therapy ceases. Targeting the HIF transcriptional system in selective cell types may provide an elegant way to overcome this survival mechanism. Furthermore, treatment of diseases characterized by insufficient angiogenesis could also benefit from a cell-specific modulation of the HIF/PHD system. Therefore, more research is required to characterize the molecular pathways initiated by the oxygen sensors.

Sources of Funding

This study was supported by the Institute for the Promotion of Innovation by Science and Technology in Flanders (Dr Coulon); Leducq Network of Excellence in Atherothrombosis (Dr Roncal); the Fund for Scientific Research in Flanders (Dr De Bock); long-term structural funding (Methusalem funding by the Flemish government) (Dr Carmeliet); interuniversity attraction pole (grant P60/30 from the Belgian government) (Dr Carmeliet); the Fund for Scientific Research in Flanders G.0692.09 (Flemish government) (Dr Carmeliet); and research grant GAO 2006/11 from the Belgian Foundation Against Cancer (Dr Carmeliet).

Disclosures

The authors disclose a possible conflict of interest (patent WO2010084134 [A1]).

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